PATENT
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Applicant:	James M. Robl et al.	Confirmation No.:	4828
Serial No.:	10/705,519	Art Unit:	1632
Filed:	November 10, 2003	Examiner:	Deborah Crouch
Customer No.:	21559		
Title:	TRANSGENIC UNGULATES HAVING REDUCED PRION PROTEIN ACTIVITY AND USES THEREOF		

DECLARATION OF DR. YOSHIMI KUROIWA TRAVERSING GROUNDS OF REJECTION OVER LACK OF ENABLEMENT

Under 37 C.F.R. § 1.132 and regarding the rejection of claims 1-6 and 25-38 for lack of enablement, I declare:

1. I am an inventor of the subject matter that is described and claimed in the above-captioned patent application. My curriculum vita was submitted with the previous Declaration submitted on September 12, 2006.

2. As described in more detail below, the specification provides ample guidance for one skilled in the art to produce hemizygous and homozygous prion protein (PrP) knockout cells and bovines. The specification, for example, at page 46, line 11 – page 47, line 31 teaches generally how knockout vectors useful for inactivating the prion locus may be constructed and further describes general methods for homologous recombination to produce knockout cells and calves.

3. The techniques utilized are then described in more detail in "EXAMPLE 1: Transgenic ungulates having reduced prion protein activity." There, at page 54, line 12 – page 61, line 17, the specification describes the actual production of hemizygous and

homozygous PrP KO bovine cells using the methods described in the specification. As stated in the specification, hemizygous $PrP^{+/-}$ cells were produced at a frequency of approximately 50%, and homozygous $PrP^{-/-}$ cells were produced from hemizygous cells at a frequency of approximately 5%.

Figs. 44A and 44B illustrate the generation of the two STOP codon-containing prion protein knockout vectors used to produce homozygous knockout cells. The specification further provides the transfection procedures employed (page 56, lines 4-26) and the methods of screening for targeted integrations (page 56, line 28 to page 57, line 25) to produce hemizygous cells. The specification then describes how to use such cells in cloning methods to produce stable cell lines (page 57, line 27 to page 58, line 26). The specification also provides methods of using the hemizygous cell lines to produce homozygous cells (page 58, line 28 to page 59, line 19). Experiments confirmed that homozygous prion protein knockout cells were produced (page 59, line 21 to page 61, line 17).

4. In addition, the specification reports that four PrP homozygous knockout cell lines (1395, 4661, 1439, and 1487-1) resulted in viable pregnancies at 90 days (page 60, Table 1). These four cell lines were derived from heterozygous PrP cell line 3560-1, which was derived from male Holstein primary fetal fibroblast line, 6594. None of the particular pregnancies reported in the specification resulted in live birth of a calf, but certain cell lines, including primary, non-cloned cell lines, do not always result in live births. To the best of my knowledge, the lack of live births using cells derived from cell line 3560-1 resulted from an animal cloning effect and was not a gene targeting error. This view is supported by the fact that we successfully produced live PrP homozygous knockout calves using the same methods and cells derived from another cell line, 3560-2, which was also derived from male Holstein primary fetal fibroblast line, 6594, and was genetically identical to 3560-1.

5. In particular, in those experiments, the male Holstein primary fetal fibroblast line, 6594, was transfected with the first and second KO vectors (pBPrP(H)KOneo and

pBPrP(H)KOpuro vectors) to sequentially disrupt the two alleles of the PrP gene as described in Kuroiwa, Y. *et al.* (Sequential targeting of the genes encoding immunoglobin-μ and prion protein in cattle. *Nat Genet.* 36: 775-780 (2004)). The methods described in Kuroiwa et al. are substantially the same as those described in the present application. Briefly, two types of KO vector were used (pBPrP(H)KOneo and pBPrP(H)KOpuro vectors) to sequentially disrupt both alleles of the PrP gene. The structure of these vectors is shown in Exhibit 1. This Exhibit is substantially the same as Figures 44A-44B of the specification. The pBPrP(H)KOneo and pBPrP(H)KOpuro vectors employed in these experiments are the same as the STOP codon-containing vectors described in the specification (at page 55, line 13 - page 56, line 2).

Fetal fibroblast line 6594 was electroporated at 550 V and 50 μF with the first KO vector (pBPrP(H)KOneo) (as substantially taught at specification page 56, lines 5-17). 94 colonies resistant to G418 (500 μg/ml) were screened by PCR to identify homologous recombinants (primer pair; neoF7 x neoR7) (40/94: 43 %) (as taught at specification page 56, line 19 to page 57, line 25). Based on their morphology, seven colonies were selected and subjected to embryonic cloning to generate fetuses (as substantially taught at specification page 58, lines 1-26). At 40-60 days of gestation, five fetuses were collected and three of them (2180, 3560-1, and 3560-2) were confirmed to be $PrP^{+/-}$ by PCR analysis (primer pair; neoF7 x neoR7).

The heterozygous $PrP^{+/-}$ cell line, 3560-2 (which, as indicated above, was genetically identical to cell line 3560-1 described in the specification), was electroporated with the second KO vector (pBPrP(H)KOpuro), and 182 colonies resistant to puromycin (1 μg/ml) were screened by PCR (primer pair; puroF14 x puroR14) to identify homozygously targeted colonies (as substantially taught at specification page 58, line 28 to page 60, line 12). Six colonies were identified to be $PrP^{-/-}$, four of which were used for embryonic cloning to generate recloned fetuses (as substantially taught at specification page 60, lines 12-21, Table 1).

At 40-75 days of gestation, 10 fetuses were collected, and fibroblast cell lines were established (as substantially taught at specification page 60, lines 12-18). All cell

lines were confirmed to be homozygous $PrP^{-/-}$ by targeting event-specific PCR analysis (puroF14 x puroR14 and neoF7 x neoR7) and prion-negative by PCR analysis (BPrPex3-F x BPrPex3-R) (as substantially taught at specification page 61, lines 1-17).

Three of the $PrP^{-/-}$ fetal cell lines (5211, 5232, and 4296) were recloned to produce calves. We obtained five (16 %), two (8.6 %), and five (26 %) calves from the cell lines 5211, 5232, and 4296, respectively, as shown in Table 1.

Table 1. Production of cloned calves from $PrP^{-/-}$ fibroblast cell lines

Genotype	Cell line ID	No. of recipients implanted	No. of calves born (%)*	No. of calves survived (%)*
PRNP$^{-/-}$	4296	19	5 (26)	5 (26)
PRNP$^{-/-}$	5211	30	7 (23)	5 (16)
PRNP$^{-/-}$	5232	21	2 (8.6)	2 (8.6)

*Percentages were calculated by dividing the number of calves by that of recipients implanted.

In the Exhibits referenced in the following sections, the PrP gene is referred to as *PRNP*.

Exhibit 2a shows the $PrP^{-/-}$ cattle at 13 months of age. Exhibit 2b shows verification of the $PrP^{-/-}$ genotype by genomic PCR on ear biopsy fibroblasts (P, positive control; N, negative control). The $PrP^{-/-}$ calf is positive in PCR analysis with puroF14 x puroR14 and neoF7 x neoR7 primers, which are specific to the targeting events at both alleles of the PrP gene. The calf is negative for wild-type alleles of PrP gene amplified with BPrPex3F x BPrPex3R primers. Collectively, all calves born proved to be $PrP^{-/-}$. Exhibit 2c shows disruption of mRNA expression by RT-PCR analysis in fibroblasts of $PrP^{-/-}$ calves. To detect PrP mRNA, PrPmF3 x PrPmR3 primers were used. These data confirmed the disruption of PrP-specific mRNA expression in $PrP^{-/-}$ calves. Exhibit 2d shows the absence of PrP^{C} protein by Western blot analysis in fibroblasts of a $PrP^{-/-}$ calf. As a positive control, a wild type (WT) calf was analyzed. As a negative control, protein extracts from mouse fibroblasts were used because the monoclonal antibody used is claimed to be specific to bovine PrP^{C} protein. Protein extracts from the wild type calf showed the presence of 33-35 kDa bovine PrP^{C} protein in size, but no positive band from

the $PrP^{-/-}$ calf was detected. As an internal positive control, its replica blot was probed with anti-β actin antibody. Exhibit 2e shows the absence of PrP^{C} protein in peripheral blood lymphocytes (PBLs) of a $PrP^{-/-}$ calf. Exhibit 2f shows the absence of PrP^{C} protein in the brainstem of $PrP^{-/-}$ calves. We detected PrP-specific bands in the wild type calves, whereas no reaction was observed in $PrP^{-/-}$ calves or negative control mouse fibroblasts. These data clearly demonstrate that the PrP gene is functionally inactivated in the $PrP^{-/-}$ calves.

6. The $PrP^{-/-}$ cattle were monitored for growth and general health status from birth up to 18 months of age. Mean birth weight was 46 kg and average daily gain was 0.91 kg/day to 10 months. Both values were in the normal range for Holstein bulls. Serum chemistry was evaluated at 6 months of age and compared with published reference ranges. All the values for $PrP^{-/-}$ calves (n = 12) were well within the reference range (Supplementary Table 1), and obvious abnormalities were not observed.

Supplementary Table 1. Serum chemistry values for $PrP^{-/-}$ cattle

Parameter	Reference Range*	$PrP^{-/-}$ calves (mean ± S.E.M.)**
Albumin (g/dl)	2.2-4.4	3.24 ± 0.04
Aspartate (U/L)	39-123	82.08 ± 2.54
Calcium (mg/dl)	8.2-12.5	9.27± 0.14
Gamma glutamyl transpeptidase (U/L)	9-42	13.42 ± 1.37
Total protein (g/dl)	5.2-8.6	6.66 ± 0.20
Globulin (g/dl)	2.1-4.9	3.43 ± 0.18
Blood urea nitrogen (mg/dl)	5-27	7.75 ± 0.39
Creatine kinase (U/L)	43-1007	265.08 ± 30.97
Phosphate (mg/dl)	4.2-10.9	9.09 ± 1.19
Magnesium (mg/dl)	1.5-2.3	2.0 ± 0.03

*International Species Information System, *Bos taurus taurus*; Domestic cow/ox; Conventional U.S.A. units

**n = 12

General physical examinations, done at monthly intervals by licensed veterinarians, included the following parameters: body temperature, heart rate, heart sound, jugular vein distension, respiratory rate, respiratory sound, presence of cough, nasal discharge, eye abnormalities, appetite, general behavior (alert and active, sluggish, hyperactive), gait, posture, joints, hooves, feces (diarrhea, constipation), genitalia, and

umbilical cord (dry, enlarged, inflamed, infected). All parameters were normal for all $PrP^{-/-}$ cattle (n = 12), and all $PrP^{-/-}$ cattle survived this observation period without any unusual health problems.

At 10 months of age, eight pairs of $PrP^{-/-}$ and age, sex, and breed-matched wild type control cattle were further given an extensive clinical examination (consisting of 122 parameters). These examinations were done according to the diagnostic evaluation of ruminants suspected of TSE (transmissible spongiform encephalopathy) as described in the European TSE guideline "Surveillance and diagnostic of TSEs in ruminants". The clinical evaluation included a general examination of all organic systems and a detailed examination of the nervous system. Examination of the nervous system focused on the following aspects: (i) evaluation of mental status, studied by observation of animal behaviour and reactions to stimulations (approaching, menace, sounds, and light); (ii) evaluation of sensory function in limbs and trunk that included the study of superficial sensitivity, medular reflexes, and conscious proprioception; (iii) evaluation of motor function in limbs and trunk by studying the muscular tone, motor irritability (presence of muscles fasciculation and tremor), and gait abnormalities; and (iv) evaluation of cranial nerves that was done by the observation of disorders in the corresponding innervated regions. All animals ($PrP^{-/-}$ and controls cattle) showed a good healthy status in the general clinical examination, and none showed significant alterations in the examination of the nervous system, except the following observation; a mild increased reaction to external stimulation (menace and sounds) was observed in 3/8 $PrP^{-/-}$ cattle compared to 1/8 control cattle. The response to external stimulation was recorded as positive when it was observed in three consecutive stimulations. This observation does not indicate an alteration of nervous system but only a mild nervous temperament, which was mainly observed in the knockout cattle.

Blood samples were taken for hematology from five pairs of $PrP^{-/-}$ and control cattle at 10 and 12 months of age. The means for various hematological parameters from the two samples were compared (Supplementary Table 2) between $PrP^{-/-}$ and control cattle and with published reference ranges. Overall, hematology analysis did not reveal obvious unusual characteristics in $PrP^{-/-}$ cattle at 10 or 12 months of age.

Supplementary Table 2. Hematology values for $PrP^{-/-}$ and control cattle

Blood Parameter	Reference Range*	$PrP^{-/-}$ calves (mean ± S.E.M.)**	Control calves (mean ± S.E.M.)**	P-value
White blood cell count ($10^3/\mu l$)	5.0-19.8	13.0 ± 1.43	10.4 ± 0.77	0.024
Red blood cell count ($10^3/\mu l$)	5.2-13.1	9.5 ± 0.36	8.6 ± 0.85	0.11
Hemoglobin (g/dl)	9.0-15.3	9.7 ± 0.07	10.2 ± 0.50	0.34
Hematocrit (%)	22.0-47.0	27.6 ± 0.35	29.1 ± 1.33	0.32
Mean corpuscular volume (fL)	30.2-70.2	29.1 ± 0.24	34.1 ± 0.49	0.0001
Mean corpuscular hemoglobin (pg/cell)	10.0-23.0	10.2 ± 0.17	11.9 ± 0.16	0.0001
Mean corpuscular hemoglobin content (g/dl)	28.0-38.1	35.2 ± 0.39	35.0 ± 0.19	0.28
Platelet count ($10^3/\mu l$)	193-582	330 ± 22.7	331 ± 24.4	0.99
Neutrophils ($10^3/\mu l$)	1.1-15.8	6.2 ± 0.81	3.0 ± 0.19	0.005
Band Neutrophils ($10^3/\mu l$)	0.08-0.58	0.02 ± 0.01	0.005 ± 0.001	0.126
Lymphocytes ($10^3/\mu l$)	2.3-10.1	5.9 ± 0.34	6.7 ± 0.29	0.12
Monocytes ($10^3/\mu l$)	0.09-1.26	0.4 ± 0.07	0.5 ± 0.09	0.851
Eosinophils ($10^3/\mu l$)	0.06-1.85	0.2 ± 0.08	0.2 ± 0.07	0.96
Basophils ($10^3/\mu l$)	0.04-0.08	0.02 ± 0.01	0.04 ± 0.04	0.582

*International Species Information System, *Bos taurus taurus*; Domestic cow/ox; Conventional U.S.A. units

**n = 5 for each group

To evaluate the impact of PrP^C deletion on calf development, we performed extensive histopathological analyses on two $PrP^{-/-}$ and two wild type cattle at 14 months of age. Representative samples of skin, nasal turbinate, lung, liver, kidney, spleen, salivary gland, thyroid gland, tonsils (pharyngeal, palatine), thymus, reticulum, rumen, omasum, abomasum, intestines (ileum, colon), adrenal gland, pancreas, urinary bladder, lymph nodes (retropharyngeal, prescapular, mesenteric, popliteal), aorta, striated muscles (heart, tongue, masseter, diaphragm, triceps, psoas major, biceps femoris), testicle (from two animals), nictitating membrane, sciatic nerve, both trigeminal nerves and ganglia, pituitary gland, spinal cord (cervical, thoracic, lumbar), one eye with its optic nerve, and the whole brain were evaluated. At least 14 sections of various areas of the brain (including obex, pons, colliculi, cerebellum, hippocampus, thalamus, and cerebral cortex) of each animal were examined by light microscopy. Two sections of spinal cord at cervical, thoracic, and lumbar regions were also evaluated by light microscopy. Neither obvious abnormalities nor significant lesions were observed for any tissue in either of the two groups.

Cells of the immune system play an important role in the pathogenesis of prion diseases, and PrP^C expression is readily detected in immune cells. Therefore, we examined the effects of PrP^C deficiency on the immune system of the $PrP^{-/-}$ cattle at 12-13 months of age. We evaluated B cell and T cell populations in peripheral blood lymphocytes (PBLs) of the $PrP^{-/-}$ cattle by flow cytometry. Exhibits 3a-3d show flow cytometry in peripheral blood lymphocytes stained with (3a) anti-IgM and anti-CD21 antibodies, (3b) anti-IgM and anti-lambda light chain antibodies, (3c) anti-CD4 and anti-CD8 antibodies, and (3d) anti-CD3 and anti-γδ T cell receptor antibodies. No differences were observed in any of these cell subsets between $PrP^{-/-}$ and wild type cattle.

To address immune-competence, the $PrP^{-/-}$ cattle were immunized with ovalbumin (OVA), a T cell-dependent antigen. Exhibit 3e shows the antibody response to OVA protein antigen in $PrP^{-/-}$ and WT cattle. Four $PrP^{-/-}$ and four WT cattle were immunized with OVA twice at day 0 (V1) and day 21 (V2), and OVA-specific IgG antibody titers at 7 days post V2 were determined. Mean antibody titers of $PrP^{-/-}$ group and WT group cattle and their SEM are shown. Statistical analysis using Student's t test showed no significant difference between $PrP^{-/-}$ and WT cattle ($p = 0.9$)

It has been shown in *Prnp*$^{-/-}$ mice that T cell proliferation and cytokine production induced by T cell mitogens is significantly affected, suggesting a role of PrP^C in T cell function. Therefore, PBLs were isolated from $PrP^{-/-}$ cattle and stimulated with anti-CD3 antibody, concanavalin A (ConA), and phytohemagglutinin (PHA). Exhibit 3f shows *in vitro* mitogenic response of T cells in $PrP^{-/-}$ and WT cattle. PBLs from four $PrP^{-/-}$ and four WT cattle were cultured with medium only (Med) or stimulated with immobilized anti-CD3 monoclonal antibody (CD3), Con A (concanavalin A), or PHA (phytohemagglutinin) mitogens for 48 hours, and proliferation was measured by ^3H thymidine incorporation. Mean of T cell response of $PrP^{-/-}$ group and WT group cattle and their SEM are shown. Statistical analysis using Student's t test showed no significant difference between $PrP^{-/-}$ and WT cattle ($p = 0.9$ for anti-CD3; $p = 0.4$ for Con A and $p = 0.7$ for PHA). In contrast to *Prnp*$^{-/-}$ mice, no difference in T cell proliferation after T cell mitogen stimulation was observed for $PrP^{-/-}$ cattle when compared to similarly treated cells from wild type cattle.

Exhibit 3g shows intracellular cytokine analysis of IFNγ expression in $PrP^{-/-}$ and WT control cattle by dual color flow cytometry. PBLs were stimulated by immobilized anti-CD3 monoclonal antibody for 72 hours, and intracellular IFNγ production was analyzed by surface CD3 and intracellular IFNγ (green) dual color immunofluorescent staining. Percentage of IFNγ+ T cells are shown in the upper right quadrant. Exhibit 3h shows *in vitro* IFNγ production by PBLs in $PrP^{-/-}$ and WT cattle. PBLs isolated from four $PrP^{-/-}$ and four WT cattle were stimulated by (i) immobilized anti-CD3 monoclonal antibody or (ii) Con A for 72 hours, and secreted IFNγ in the culture supernatant was analyzed by calibrated bovine IFNγ ELISA. Mean of the IFNγ production (ng/ml) in $PrP^{-/-}$ group and WT control cattle and their SEM are shown. Statistical analysis using Student's t test showed no significant difference between $PrP^{-/-}$ and WT cattle ($p = 0.5$). These data indicate that the ablation of PrP^{C} expression does not appear to have deleterious effects on the immune systems in cattle.

The $PrP^{-/-}$ bulls reached sexually maturity at a normal age, and semen was collected from two KO animals at 16 months of age to address fertility of the $PrP^{-/-}$ cattle. Sperm appeared morphologically normal and were capable of generating normal-appearing blastocysts by *in vitro* fertilization with oocytes derived from wild type cows. This result indicates that $PrP^{-/-}$ bulls are reproductively normal and could be used for production of a population of $PrP^{-/-}$ cattle for commercial application.

Collectively, these data indicate that disruption of normal prion protein PrP^{C} expression using methods as substantially described in the specification did not in any significant way adversely affect normal bovine development or health. $PrP^{-/-}$ cattle remained healthy for at least 18 months after birth.

7. In my opinion, the specification provides ample guidance for one skilled in the art to produce hemizygous and homozygous PrP knockout cells and bovines. The methods described in the specification have been successfully employed to produce both bovine cells and surviving calves. The frequency of success is also sufficient to allow one skilled in the art to produce cells or calves reproducibly in 6 months for

homozygously targeted cells or 15 months for homozygously targeted calves, after completion of vector construction.

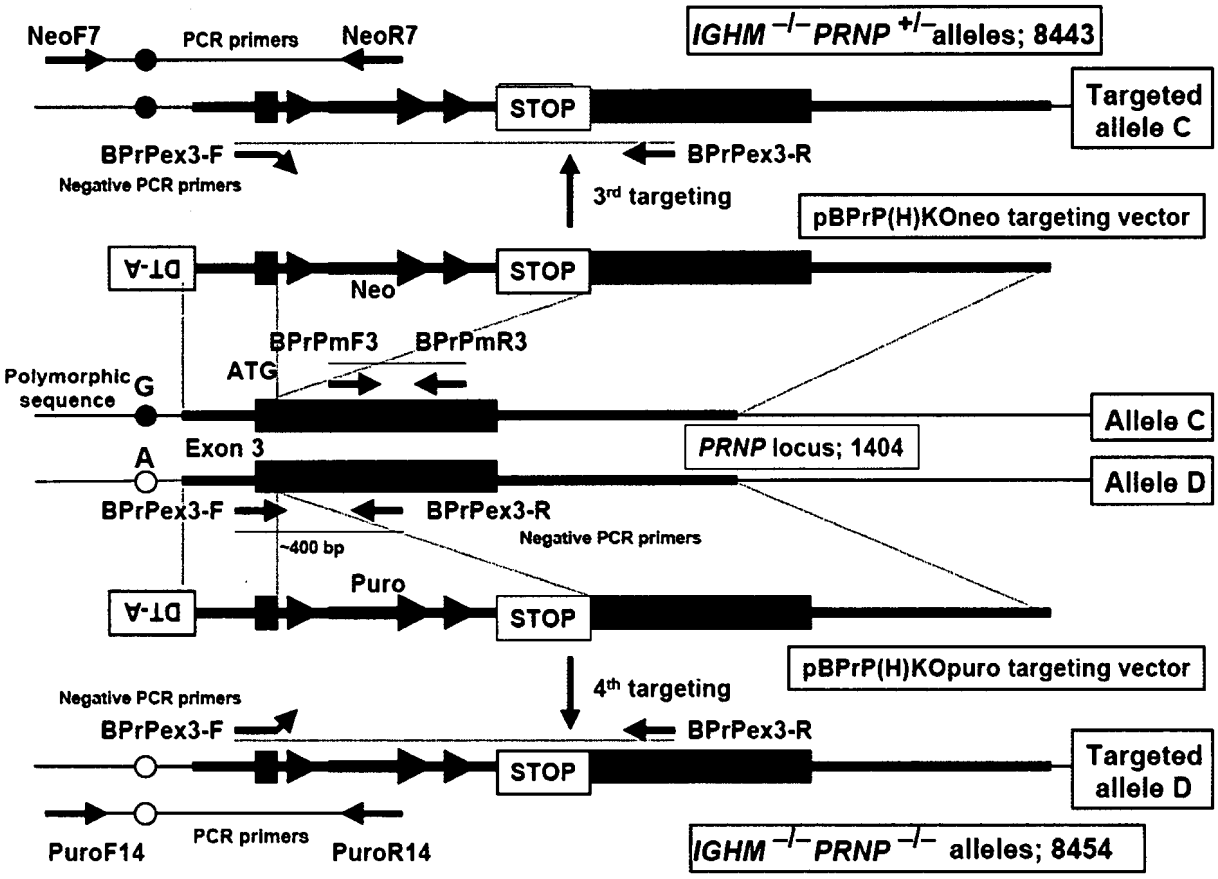
8. Further, it is my opinion that the particular cell lines actually used to successfully produce the homozygous prion knockout calves are not critical to production of prion knockout bovines. This is supported by experiments described in the specification at page 57, lines 20-25. There, the specification states, "The high frequency of homologous recombination of more than 50% was reproduced in another fibroblast cell line, female F1 (Holstein x Jersey) cell line (Fig. 44C). Thus, bovine fibroblast cell lines in which one allele of the prion locus is mutated by the knockout vector were successfully generated, irrespective of the initial cell line used."

9. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

30 May 2007
Date

Name: Dr. Yoshimi Kuroiwa

Exhibit 1


NeoF7
PCR primers
NeoR7
IGHM −/− PRNP +/− alleles; 8443
STOP
Targeted allele C
BPrPex3-F
Negative PCR primers
BPrPex3-R
3rd targeting
pBPrP(H)KOneo targeting vector
DT-A
Neo
STOP
BPrPmF3
BPrPmR3
ATG
Polymorphic sequence
G
Exon 3
A
PRNP locus; 1404
Allele C
Allele D
BPrPex3-F
BPrPex3-R
Negative PCR primers
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Puro
DT-A
STOP
pBPrP(H)KOpuro targeting vector
4th targeting
Negative PCR primers
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PCR primers
PuroR14
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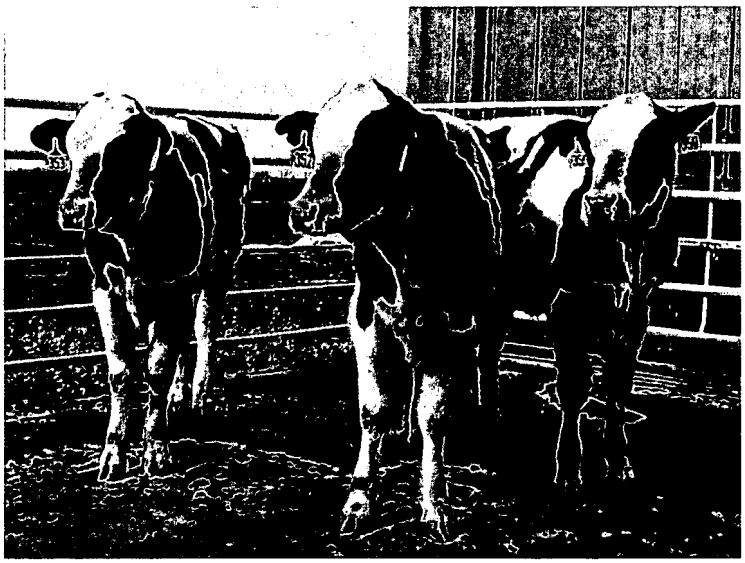
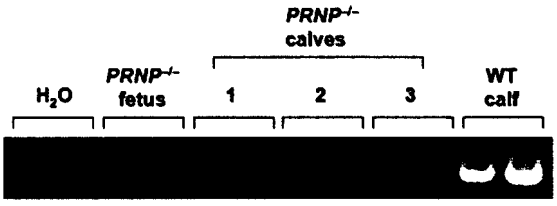
Exhibit 2

a



b

c



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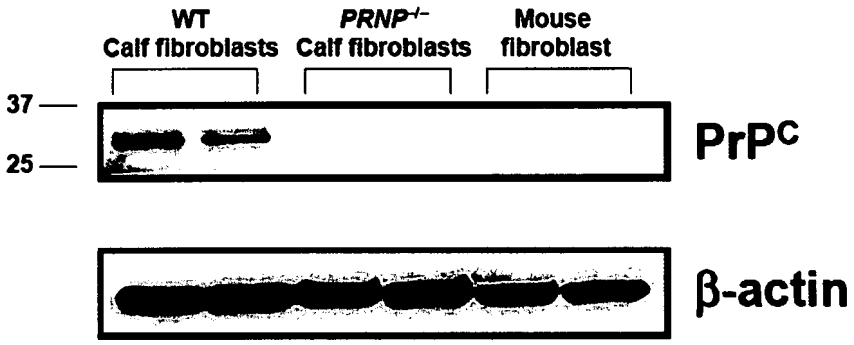
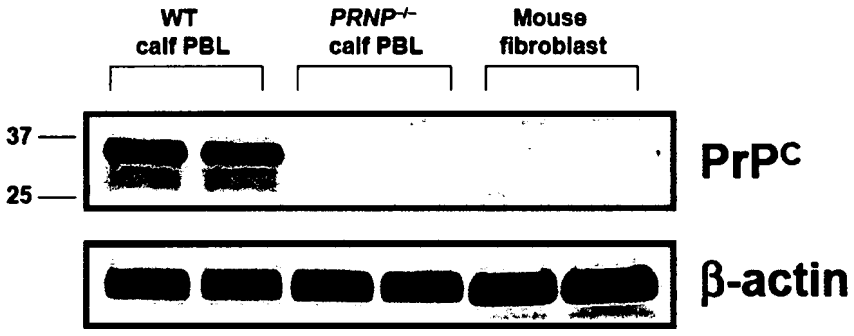
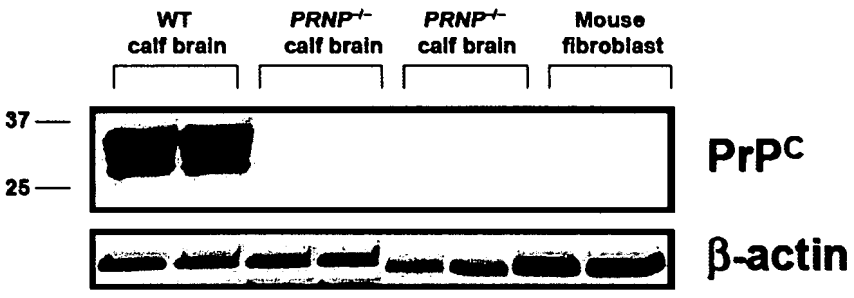

WT
Calf fibroblasts
PRNP+/-
Calf fibroblasts
Mouse
fibroblast
37
25
PrPC
β-actin

e


WT
calf PBL
PRNP+/-
calf PBL
Mouse
fibroblast
37
25
PrPC
β-actin

f


WT
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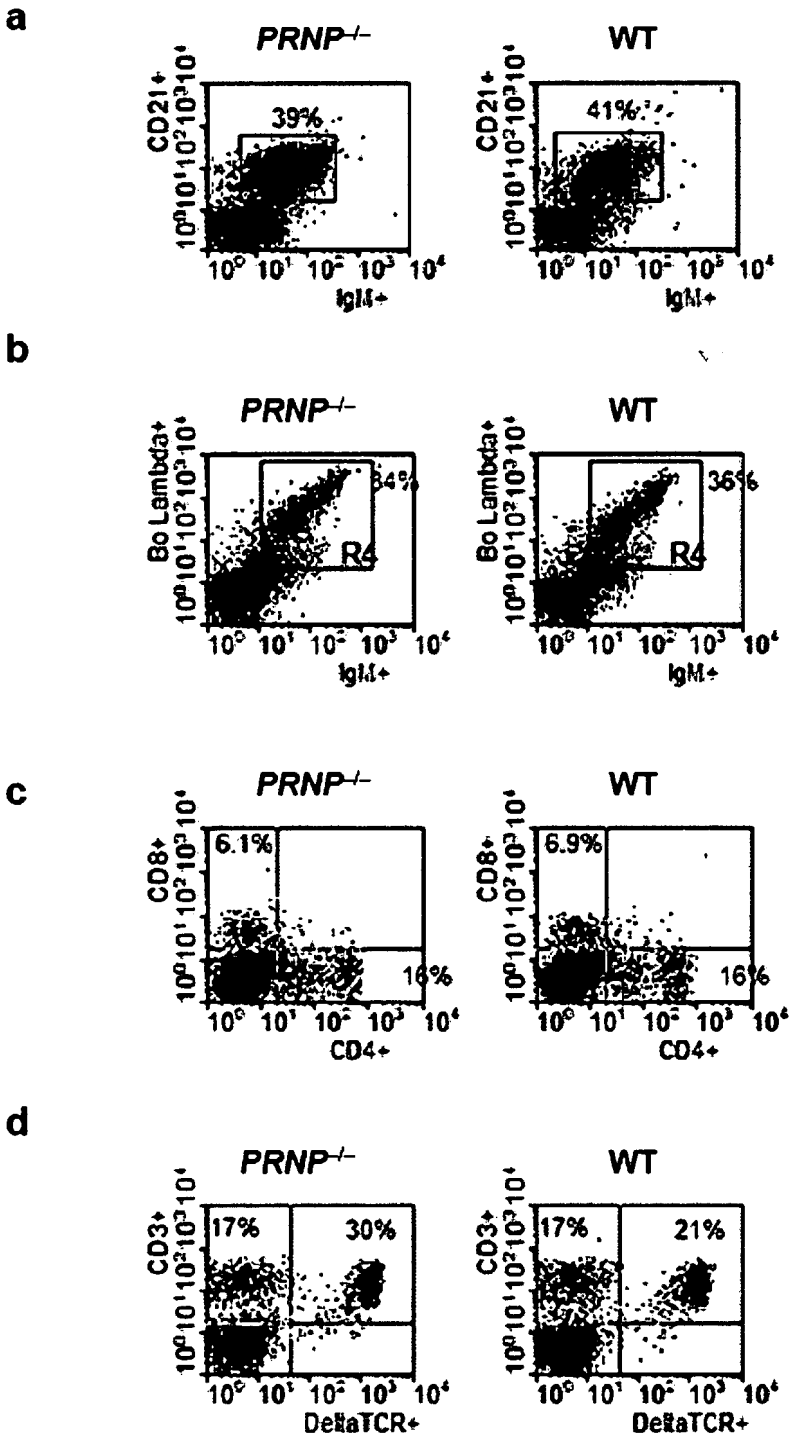
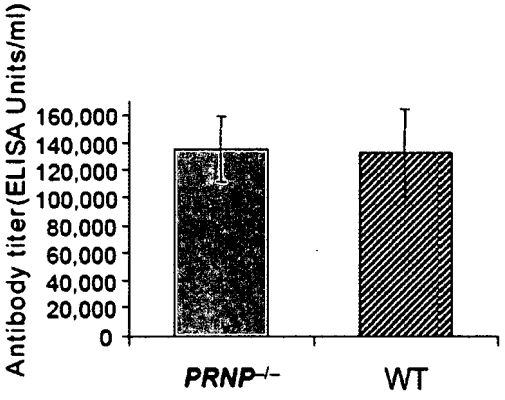
PRNP+/-
calf brain
PRNP+/-
calf brain
Mouse
fibroblast
37
25
PrPC
β-actin

Exhibit 3


a
PRNP-/-
WT
CD21+
39%
41%
IgM+
b
PRNP-/-
WT
Bo Lambda+
R4
IgM+
c
PRNP-/-
WT
CD8+
6.1%
6.9%
16%
CD4+
d
PRNP-/-
WT
CD3+
17%
30%
21%
DeltaTCR+

e


Antibody titer(ELISA Units/ml)
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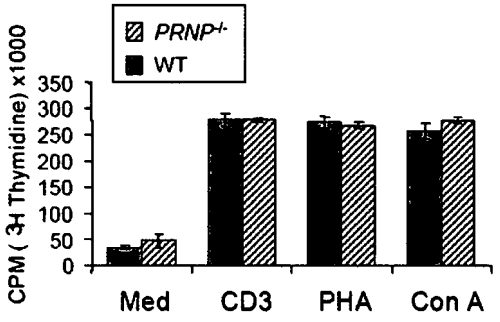
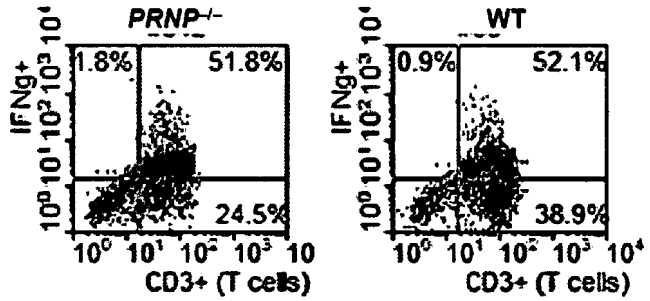
120,000
100,000
80,000
60,000
40,000
20,000
0
PRNP-/-
WT

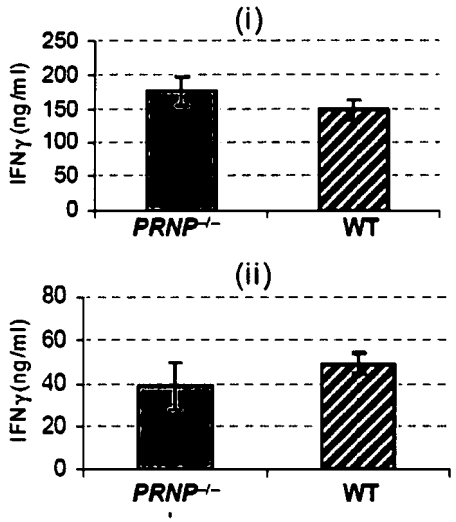
f


PRNP-/-
WT
CPM (3H Thymidine) x1000
350
300
250
200
150
100
50
0
Med
CD3
PHA
Con A

g


PRNP-/-
WT
IFNg+
1.8%
51.8%
24.5%
0.9%
52.1%
38.9%
CD3+ (T cells)

h


(i)
250
200
150
100
50
0
IFNγ(ng/ml)
PRNP-/-
WT
(ii)
80
60
40
20
0
IFNγ(ng/ml)
PRNP-/-
WT